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Pharmacological characterization of desensitization in a human mGlu1α-expressing non-neuronal cell line co-transfected with a glutamate transporter

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1 Stimulation of phosphoinositide hydrolysis by human mGlu1α (HmGlu1α) was examined in a non-neuronal cell line (AV12-664) co-expressing both HmGlu1α and a rat glutamate/aspartate transporter (GLAST).

2 Desensitization of HmGlu1α could be elicited by inhibition of the GLAST transporter with the glutamate uptake inhibitor, L-*trans*-pyrrolidine-2,4-dicarboxylic acid (*trans*-PDC). Maximal inhibition of HmGlu1α-mediated phosphoinositide hydrolysis was induced upon 24 h pretreatment with *trans*-PDC. The concentration of glutamate in the extracellular medium also rose significantly in cells pretreated with *trans*-PDC. Glutamate levels increased upon incubation with *trans*-PDC in a time-dependent manner, with maximal glutamate levels attained after 24 h incubation with *trans*-PDC.

3 The time required for desensitization of HmGlu1α by *trans*-PDC was compared to the time course for desensitization elicited by the direct-acting mGlu receptor agonists, 1-aminocyclopentane-1**S**,3**R**-dicarboxylic acid (1**S**,3**R**-ACPD) and (**R**,**S**)-3,5-dihydroxyphenylglycine (3,5-DHPG). Both direct-acting mGlu receptor agonists elicited desensitization of HmGlu1α more rapidly than did *trans*-PDC, with maximal inhibition of agonist-induced phosphoinositide hydrolysis upon 12 h pretreatment. Agonist-induced desensitization could be fully reversed upon washout of agonist for 12 h.

4 Both mGlu receptor agonist- and *trans*-PDC-induced desensitization of HmGlu1α could be blocked by inclusion of (+)-α-methyl-4-carboxyphenylglycine (MCPG), an mGlu receptor antagonist, in the pretreatment medium.

5 Agonist-stimulated phosphoinositide hydrolysis by HmGlu1α was found to parallel closely agonist-induced desensitization of HmGlu1α. Thus, the EC_{50} values for 1**S**,3**R**-ACPD- and 3,5-DHPG-stimulated phosphoinositide hydrolysis were similar to the EC_{50} values for eliciting desensitization of HmGlu1α.

6 These studies demonstrate desensitization of recombinant human mGlu1α receptor in a non-neuronal cell line in which the receptor can be regulated by direct activation or by manipulation of glutamate transporter activity. Desensitization of HmGlu1α was found to be mediated by activation of the receptor since the mGlu receptor antagonist, MCPG, blocked both mGlu receptor agonist- and *trans*-PDC-induced desensitization of HmGlu1α. Furthermore, agonist-induced desensitization of HmGlu1α was found to parallel receptor-mediated stimulation of phosphoinositide hydrolysis.

Keywords: Metabotropic glutamate receptors; receptor down-regulation; phosphoinositide hydrolysis; glutamate uptake; GLAST transporter; inositol-1,4,5-trisphosphate; AV12-664 cell line; 1**S**,3**R**-ACPD; *trans*-PDC; mGluR

Introduction

Receptor-mediated desensitization has been demonstrated for a variety of G-protein-coupled receptors that are linked to the phosphoinositide second messenger system. This is thought to occur by a number of mechanisms that are initiated upon agonist activation of the receptor (for review, see Wojcikiewicz *et al.* 1993). Since activation of phosphoinositide hydrolysis elicits formation of diacylglycerol (DAG) and subsequent activation of protein kinase C (PKC), regulation of phosphoinositide-coupled receptors may involve a rapid, negative feedback mechanism in which receptor uncoupling is due to phosphorylation by PKC (Nishizuka, 1988). Another mechanism that has been demonstrated for receptors coupled to phospholipase C (PLC) involves internalization of the receptor into vesicles, resulting in a loss of receptors from the cell surface (Thompson & Fisher, 1990; Lameh *et al.*, 1992). In addition to regulation at the receptor level, desensitization of receptor function may also involve down-regulation of the G protein (Milligan, 1993), specific isoforms of PKC (Kiley *et al.*, 1991), or the inositol 1,4,5-trisphosphate ($InsP_3$) receptor (Wojcikiewicz *et al.*, 1992; Simpson *et al.*, 1994).

Metabotropic (G protein-coupled) glutamate (mGlu) receptors coupled to phosphoinositide hydrolysis have been shown to undergo desensitization in a number of experimental systems. For instance, rapid desensitization of mGlu receptor-mediated phosphoinositide hydrolysis has been demonstrated in a number of *in situ* preparations, including rat brain slices (Schoepp & Johnson, 1988; Godfrey & Taghavi, 1990), and neuronal cultures (Canonico *et al.*, 1988; Ambrosini & Meldolesi, 1989; Catania *et al.*, 1991; Aronica *et al.*, 1993). This rapid desensitization of mGlu receptors coupled to phosphoinositide hydrolysis is thought to be mediated by activation of PKC; however, these studies are limited by the fact that *in situ* preparations do not readily allow the study of a receptor subtype in isolation. Recently however, a number of mGlu receptors have been cloned, thereby allowing the study of a single recombinant mGlu receptor. Phosphoinositide-coupled mGlu receptors include mGlu1α (Houamed *et al.*, 1991; Masu *et al.*, 1991) and two splice variants, mGlu1β (Tanabe *et al.*, 1992) and mGlu1c (Pin *et al.*, 1992), as well as mGlu5a (Abe *et al.*, 1992) and mGlu5b (Minakami *et al.*, 1993). Recent studies have explored the desensitization phenomenon in more detail using cloned mGlu receptors expressed in cell lines. For instance, rapid desensitization of mGlu receptor-mediated phosphoinositide hydrolysis has been demonstrated upon activation of PKC with phorbol esters in mGlu1α-expressing baby hamster kidney (BHK) cells (Thomsen *et al.*, 1993). In-

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deed, Alaluf *et al.* (1995) have recently shown that application of mGlu receptor agonists to mGlu1α-expressing BHK cells elicits rapid and transient phosphorylation of the receptor, an effect mimicked by phorbol esters and blocked by PKC inhibitors, thus further supporting the hypothesis that rapid desensitization of mGlu1α involves a PKC-dependent pathway. In cultured cerebellar granule neurones, desensitization of mGlu1 has been shown to result in reduced levels of mGlu1 mRNA upon exposure to the mGlu receptor agonists, glutamate and quisqualate (Bessho *et al.*, 1993), or in the presence of high K^+ (Favaron *et al.*, 1992; Bessho *et al.*, 1993; Aronica *et al.*, 1993).

We recently reported the cloning and expression of human mGlu1α (HmGlu1α) in AV12-664 cells co-expressing a rat glutamate/aspartate transporter (GLAST; Storck *et al.*, 1992; Desai *et al.*, 1995). We found that HmGlu1α receptor-mediated phosphoinositide hydrolysis was enhanced in cells co-expressing the rat GLAST transporter (RGT/HmGlu1α cells). This is probably due to removal of glutamate from the extracellular medium by the GLAST transporter. However, long-term exposure to the glutamate uptake inhibitor, L-*trans*-pyrrolidine-2,4-dicarboxylic acid (*trans*-PDC), resulted in a significant reduction of HmGlu1α-mediated phosphoinositide hydrolysis. Long-term blockade of the GLAST transporter was found to increase levels of glutamate in the extracellular medium, presumably resulting in chronic activation and subsequent desensitization of HmGlu1α. In the present studies, we further investigated the hypothesis that increased levels of receptor agonist (i.e. endogenous glutamate) following inhibition of glutamate uptake results in subsequent loss of mGlu receptor agonist-evoked phosphoinositide hydrolysis via receptor-mediated desensitization. This was determined by examining (1) the ability of direct-acting mGlu1α agonists to desensitize receptor responses; (2) the temporal requirements for desensitization induced by *trans*-PDC versus direct-acting mGlu1α receptor agonists; and (3) the effects of the competitive mGlu receptor antagonist, (+)-α-methyl-4-carboxyphenylglycerine (MCPG), on agonist-evoked desensitization.

Methods

The RGT/HmGlu1α cell line was created and maintained as previously described (Desai *et al.*, 1995). Briefly, AV12-664 cells (ATCC, CRL 9595) were transfected with rat GLAST cDNA (Storck *et al.*, 1992) in a mammalian expression vector, pRS/RSV (Invitrogen). These cells, referred to as RGT cells, were subsequently transfected with HmGlu1α cDNA in the pGT-h expression vector (Berg *et al.*, 1993). Transfections of the plasmids into cells were carried out by a modified calcium phosphate precipitation method (Graham & Van Der Eb, 1973) with reagents obtained from Stratagene, Inc.: 10 μg of plasmid were used without carrier DNA for each 10 cm petri plate of cells at approximately 50% confluency. Clones expressing GLAST were selected by resistance to G-418 (500 μg ml^{-1}) (Gibco BRL). HmGlu1α-expressing clones were selected by resistance to hygromycin (250 μg ml^{-1}). Resistant clones were tested for HmGlu1α expression by measurement of agonist-stimulated phosphoinositide hydrolysis. RGT/HmGlu1α cells were grown in Dulbecco's modified Eagle's medium supplemented with 1.2 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 5% dialyzed foetal calf serum, 250 μg ml^{-1} hygromycin, and 500 μg ml^{-1} G-418. Cells were maintained in a 6.8% CO_2 incubator at 37°C.

To measure phosphoinositide hydrolysis, cells were plated in 24-well plates and grown for 1–4 days in normal growth medium. This was followed by a medium change in which 1 ml of fresh medium containing 4μCi ml^{-1} *myo*-[2-^{3}H]-inositol (Amersham) was added to each well. Pretreatment of cells with agonists, the antagonist MCPG, or the uptake blocker *trans*-PDC was performed at various time points by addition of the compound to the medium. To determine reversal of agonist-induced desensitization by washout of agonist from the medium, agonists were added to the medium for 24 h. Medium was removed from the wells, the cells were washed with Hank's Balanced Salts Solution (GIBCO BRL), and 1 ml of fresh medium containing 4 μCi ml^{-1} *myo*-[2-^{3}H]-inositol was added to each well for 12–14 h before conducting the assay. Some wells received agonist again during the incubation with *myo*-[2-^{3}H]-inositol to prevent reversal of desensitization.

After incubation of cells in *myo*-[2-^{3}H]-inositol for 12–14 h, medium was removed and the cells were washed 2–3 times with DMEM containing 10 mM *myo*-inositol, and 10 mM HEPES. For measurement of phosphoinositide hydrolysis, cells were incubated in DMEM containing 10 mM *myo*-inositol, 10 mM LiCl, and 10 mM HEPES. Agonists were added to the medium, and cells were incubated at 37°C for 1 h under a CO_2 atmosphere. At the end of the incubation, the reaction was stopped by placing the plates on ice, quickly removing the incubation medium, and adding 1 ml acetone:methanol (1:1). Stimulation of phosphoinositide hydrolysis was assayed by measuring the accumulation of [^{3}H]-inositol monophosphate ([^{3}H]-InsP). [^{3}H]-InsP was isolated by QMA Sep-Pak anion column chromatography (Millipore Corp.) by elution with triethylammonium bicarbonate (Fluka Chemicals) (Maslanski & Busa, 1990). Data were calculated as d.p.m. of [^{3}H]-InsP per milligram of protein and converted to a percentage of the basal [^{3}H]-InsP value in each experiment. Protein content in each well was determined by a modified Bradford-Pierce assay (Pierce Chemicals).

To determine the levels of glutamate released into the extracellular medium by RGT/HmGlu1α cells, cells were plated in 24-well plates, and fresh medium was added when the cells were approximately 50% confluent. Cells were incubated for 24 h (or 48 h for the 48 h point), and 300 μM *trans*-PDC or vehicle was added at varying time points. Samples of medium were collected and diluted with volumes of 0.01 N HCl to give concentrations in the range of glutamate standard solutions (0.1–10 μM). The samples were mixed with equal volumes of Fluo-R fluorescence reagent (Beckman Instruments). The mix was kept at room temperature for 1 min to derivatize the sample before being injected into a 20 μl loop by a Beckman Autosampler 507. The column used was an Ultrasphere ODS C18 5 μM column (2 mm × 25 mm) at 30°C. The h.p.l.c. system consists of a high-pressure pump (Beckman System Gold) with a flow rate of 0.3 ml in conjunction with a fluorescent detector (Jasco, FP-920; excitation/emission wavelengths: 360/450 nm). The mobile phase consisted of (A) 50 mM sodium phosphate (pH 7.2) containing 10% methanol and (B) 50 mM sodium phosphate (pH 7.2) containing 70% methanol. Gradient elution involved use of 98% A/2%B initially, then increasing to 4%B over 20 min. Mobile phase B was increased to 98% over 3 min and maintained for 25 min to elute other substances, then returned to the initial conditions for 15 min before running the next sample. Based on comparison to standards, glutamate eluted at 16 min.

Statistical analysis

To determine EC_{50} values from concentration-response curves on [^{3}H]-InsP accumulation, the median-effect plot of Chou & Talalay (1983) was used. Statistical significance was determined by using two-way ANOVA in conjunction with least squares means. Values of $P < 0.05$ were considered significantly different from control.

Materials

The following items were purchased from Tocris Cookson: 1-aminocyclopentane-1**S**,3**R**-dicarboxylic acid (1**S**,3**R**-ACPD), (+)-α-methyl-4-carboxyphenylglycine (MCPG), and L-*trans*-pyrrolidine-2,4-dicarboxylic acid (*trans*-PDC). (**R**,**S**)-3,5-dihydroxyphenylglycine (3,5-DHPG) was supplied by S. Richard Baker (Lilly Research Centre; Windlesham, U.K.). All materials for cell culture media were purchased from GIBCO BRL.

Results

We have previously shown that long-term pretreatment of RGT/HmGlu1α cells with the glutamate uptake inhibitor, *trans*-PDC, results in an increase in glutamate concentrations in the extracellular medium, with subsquent desensitization of HmGlu1α (Desai *et al.*, 1995). In the present studies, the time-course required for *trans*-PDC-induced desensitization of HmGlu1α was examined. RGT/HmGlu1α cells were pretreated with *trans*-PDC for 6, 12, 24, or 48 h. At the end of the pretreatment period, the cells were washed several times to remove *trans*-PDC, and agonist-stimulated phosphoinositide hydrolysis was measured. As shown in Figure 1a, 6 h pretreatment with *trans*-PDC did not significantly decrease glutamate- or 1S,3R-ACPD-stimulated phosphoinositide hydrolysis. In contrast, 12 h pretreatment resulted in a significant decrease of ACPD-stimulated phosphoinositide hydrolysis, but not in glutamate-stimulated phosphoinositide hydrolysis. Upon pretreatment with *trans*-PDC for 24 h, both glutamate- and ACPD-stimulated phosphoinositide hydrolysis were decreased by approximately 50%, and the response was not further inhibited by pretreatment for 48 h. These data suggest that maximal desensitization of HmGlu1α is attained by 24 h pretreatment with *trans*-PDC.

In order to determine if the degree of desensitization was dependent on the concentration of glutamate in the extracellular medium, the concentration of glutamate attained upon incubation with 300 μM *trans*-PDC for 6, 12, 24, or 48 h was determined. RGT/HmGlu1α cells were incubated in fresh medium for 24 h (or 48 h in the case of the cells receiving *trans*-PDC for 48 h), and 300 μM *trans*-PDC or water vehicle was added at various times during the incubation. H.p.l.c. analysis of glutamate levels showed that glutamate levels were normally at or below 1 μM in the extracellular medium after 24 or 48 h (Figure 1b). In contrast, the concentration of glutamate in the extracellular medium collected from cells receiving 300 μM *trans*-PDC increased in a time-dependent manner up to levels >50 μM (Figure 1b), which are sufficient to activate these receptors (Desai *et al.*, 1995). Interestingly, glutamate levels were not significantly different in the medium of cells incubated in *trans*-PDC for 24 versus 48 h. These data support the hypothesis that desensitization of HmGlu1α by *trans*-PDC is dependent on the concentration of glutamate attained in the extracellular medium.

Desensitization of HmGlu1α by *trans*-PDC is presumably a two-step process that requires blockade of glutamate transport followed by chronic activation of HmGlu1α. In the next series of studies, we determined if desensitization of HmGlu1α could be elicited upon long-term pretreatment of RGT/HmGlu1α cells with direct-acting mGlu receptor agonist, thereby eliminating the need for accumulation of glutamate in the extracellular medium. The agonists 3,5-DHPG and 1S,3R-ACPD maximally stimulate phosphoinositide hydrolysis, but do not inhibit glutamate uptake by the GLAST transporter in RGT/HmGlu1α cells, suggesting that they are selective for HmGlu1α in these cells (Desai *et al.*, 1995). The time-course for desensitization of HmGlu1α by these agonists was thus examined. Unlike *trans*-PDC, 1S,3R-ACPD (300 μM) added to RGT/HmGlu1α cells for 24 h did not substantially alter the concentration of glutamate in the extracellular medium (control, 1.3±0.1 μM; 1S,3R-ACPD, 1.7±0.1 μM; $n=3$). As shown in Figure 2, 6 h pretreatment with direct-acting mGlu receptor agonists resulted in a small, but significant, reduction in glutamate- and 1S,3R-ACPD-stimulated phosphoinositide hydrolysis. 3.5-DHPG and 1S,3R-ACPD elicited maximal inhibition of agonist-stimulated phosphoinositide hydrolysis after only 12 h pretreatment (Figure 2).

To determine if agonist-induced desensitization of HmGlu1α could be reversed, we determined the effect of washing out agonist from the cells on subsequent glutamate-induced phosphoinositide hydrolysis. RGT/HmGlu1α cells received 100 μM 3,5-DHPG or 300 μM 1S,3R-ACPD for 24 h, followed by washout of the medium and addition of fresh medium for 12–14 h. Some wells received agonist during the washout period to prevent reversal of desensitization. As Figure 3 shows, 12 h washout of either 3,5-DHPG or 1S,3R-ACPD completely reversed agonist-induced desensitization of HmGlu1α-mediated phosphoinositide hydrolysis. Interestingly, the time required to reverse agonist-induced desensitization appears to be similar to that required to elicit

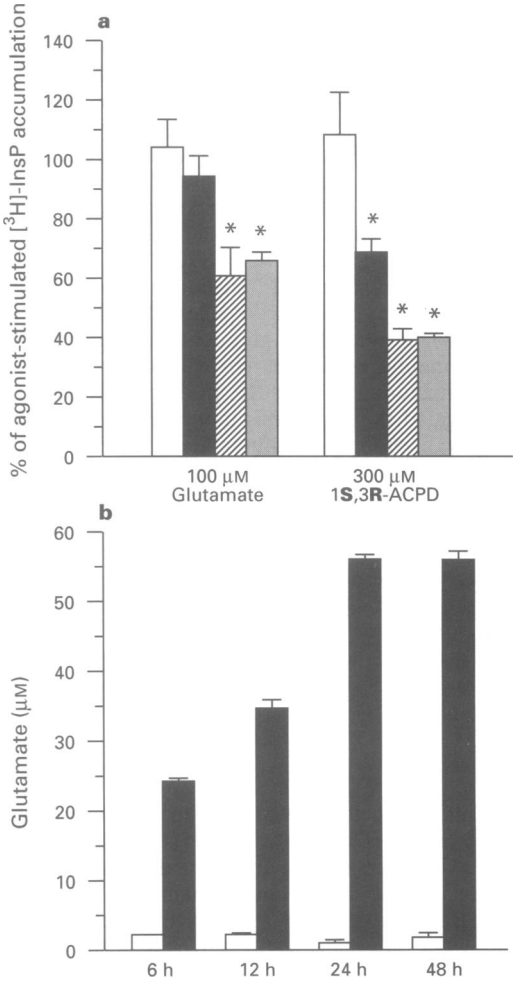

Figure 1 Effect of varying pretreatment times with *trans*-PDC on subsequent agonist-induced phosphoinositide hydrolysis and glutamate concentrations in RGT/HmGlu1α cells. (a) Cells were prelabelled with [^{3}H]-inositol for 24 h and simultaneously incubated for 6 h (open columns), 12 h (solid columns), 24 h (hatched columns), or 48 h (stippled columns) with 300 μM *trans*-PDC or water (100% control value) in the culture medium. At the end of the pre-incubation, the cells were washed three times with fresh medium to remove *trans*-PDC. The formation of [^{3}H]-InsP (in the presence of LiCl) was determined in response to 100 μM glutamate of 300 μM 1S,3R-ACPD. Data are expressed as percentage of agonist-stimulated accumulation of [^{3}H]-InsP in control cells pretreated with water vehicle. Each column represents a mean (with s.e.mean) of 3 separate experiments, except the 6 h time point which represents a mean of 4 experiments, each done in triplicate. Absolute values for all time points are 35,380±1500 d.p.m. mg^{-1} protein for basal, 527,270±30,260 d.p.m. mg^{-1} protein for 100 μM glutamate, and 386,040±22,400 d.p.m. mg^{-1} protein for 300 μM 1S,3R-ACPD. *$P<0.05$ when compared to control (100%). (b) RGT/HmGlu1α cells at approximately 50% confluency were incubated in fresh medium for 24 h (6, 12, or 24 h time points) or 48 h (48 h time point). At various times during the incubation, cells received water vehicle (control, open columns) or 300 μM *trans*-PDC (solid columns). Medium fron the wells was collected, and glutamate concentrations were determined by h.p.l.c. analysis. Glutamate levels were determined for 3 experiments, each done in triplicate.

desensitization since we saw partial, but not complete, reversal of 3,5-DHPG-induced desensitization after 6 h washout of agonist (data not shown).

In order to ensure that the desensitizing effects of *trans*-PDC or direct-acting agonists are mediated by activation of HmGlu1α, we determined if the mGlu1 receptor antagonist, MCPG, would block desensitization of agonist-stimulated phosphoinositide hydrolysis by *trans*-PDC or by HmGlu1α receptor agonists. Twenty-four hour incubation with either 300 μM *trans*-PDC or 1 mM MCPG did not affect basal phosphoinositide hydrolysis. Pretreatment with *trans*-PDC reduced glutamate-stimulated phosphoinositide hydrolysis (control) by 31%, although this was not significant at the $P<0.05$ level ($P=0.085$). Nevertheless, the phosphoinositide hydrolysis response to MCPG in the presence of *trans*-PDC was significantly different from the response to *trans*-PDC alone and was not significantly different from the control value (Figure 4). Similarly, pretreatment with the direct-acting mGlu1 receptor agonists 3,5-DHPG and 1S,3R-ACPD significantly inhibited glutamate-stimulated phosphoinositide hydrolysis, and co-application of MCPG blocked the inhibition (Figure 5). These data support the hypothesis that agonist-induced desensitization of HmGlu1α is a receptor-mediated event.

To determine the potency of the direct-acting agonists for inducing desensitization of HmGlu1α, RGT/HmGlu1α cells were pretreated for 24 h with increasing concentrations of 3,5-DHPG or 1S,3R-ACPD. Glutamate-stimulated phosphoinositide hydrolysis was determined in vehicle-treated cells and in cells incubated for 24 h with increasing concentrations of agonist. As shown in Figure 6, pretreatment with either of the agonists caused a concentration-dependent desensitization of glutamate-stimulated phosphoinositide hydrolysis in RGT/HmGlu1α cells. A comparison of the EC_{50} for desensitization of HmGlu1α by pretreatment with these agonists was found to

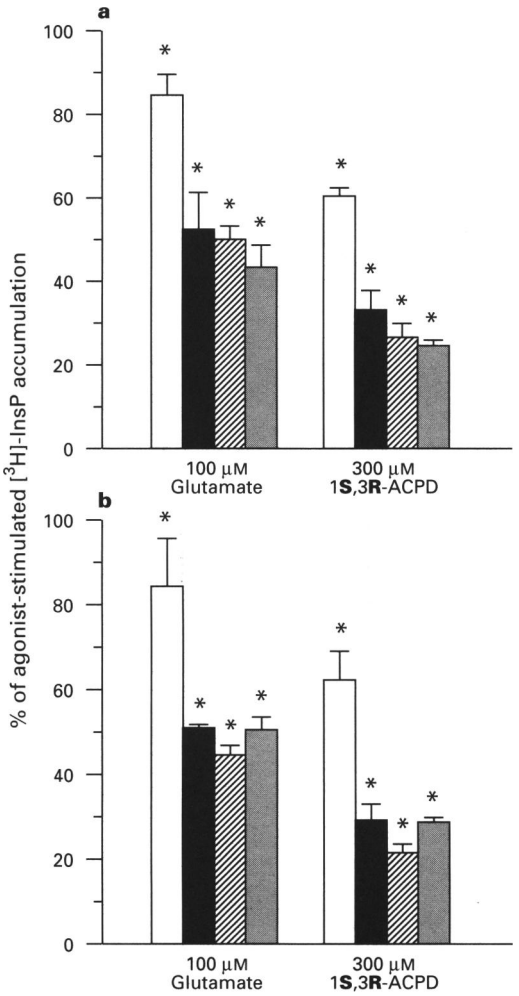

Figure 2 Effect of pretreatment with 3,5-DHPG (a) or 1S,3R-ACPD (b) on glutamate- or 1S,3R-ACPD-stimulated phosphoinositide hydrolysis in RGT/HmGlu1α cells. Cells were prelabelled with [^{3}H]-inositol for 24 h and simultaneously incubated for 6 h (open columns), 12 h (solid columns), 24 h (hatched columns), or 48 h (stippled columns) with agonist (100 μM DHPG, a; 300 μM 1S,3R-ACPD, b) or water (100% control value) in the culture medium. Measurement of phosphoinositide hydrolysis was conducted as in Figure 1. Each column represents a mean (with s.e.mean) of 3 separate experiments, each done in triplicate. *$P<0.05$ when compared to control (100%).

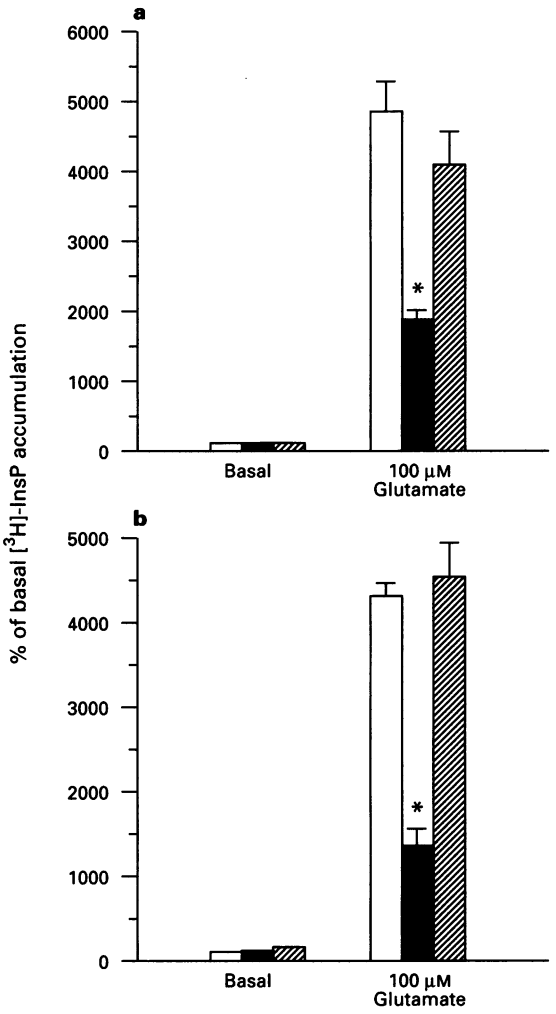

Figure 3 Effect of pretreament with 3,5-DHPG (a) or 1S,3R-ACPD (b) and subsequent washout of agonist to reverse desensitization of HmGlu1α. Agonists or water vehicle were added for 24 h, the cells were washed and fresh medium containing myo-[^{3}H]-inositol was added to each well, and incubation proceeded for an additional 12–14 h. Some cells received agonist during this incubation as well to prevent reversal of desensitization (total incubation with agonist in these cells was approximately 36 h). At the end of this incubation, medium was removed and phosphoinositide hydrolysis was measured as in Figure 1. Open columns represent control values, solid columns represent agonist pretreatment for 36 h (3,5-DHPG, a; 1S,3R-ACPD, b), and cross-hatched columns represent agonist pretreatment for 24 h followed by washout of the agonist and further incubation for 12–14 h (3,5-DHPG, a; 1S,3R-ACPD, b). Each column represents a mean (with s.e.mean) of 3 separate experiments, each done in triplicate, except the basal value for 100 μM DHPG or 300 μM 1S,3R-ACPD which were both a mean of 2 separate experiments, in triplicate. *$P<0.05$ when compared to control value for 100 μM glutamate-stimulated phosphoinositide hydrolysis.

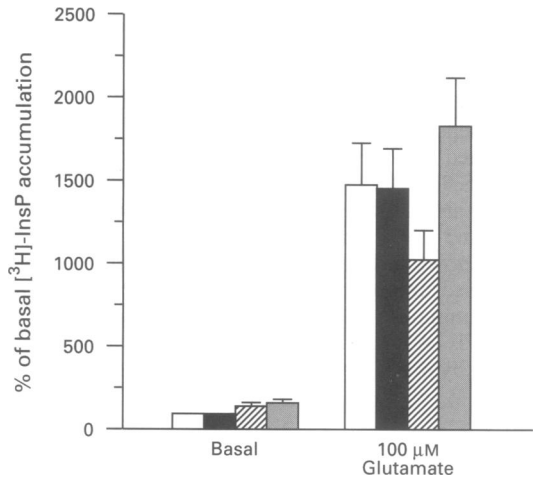

Figure 4 Effect of MCPG pretreatment on *trans*-PDC-induced desensitization of glutamate-stimulated phosphoinositide hydrolysis in RGT/HmGlu1α cells. Cells were pretreated with water vehicle (control, open column), 1 mM MCPG (solid column), 300 μM *trans*-PDC (hatched column), or both compounds (stippled column), for 24 h. At the end of this incubation period, the cells were washed to remove *trans*-PDC. Measurement of glutamate-stimulated phosphoinositide hydrolysis was conducted as in Figure 1. Each column represents a mean (with s.e.mean) of 6 separate experiments, each done in triplicate.

match closely agonist EC_{50}s for acute stimulation of HmGlu1α-mediated phosphoinositide hydrolysis (Table 1) (Desai *et al.*, 1995).

Discussion

The study of the properties of metabotropic glutamate receptor subtypes has been greatly enhanced with the expression of recombinant receptors in immortal cell lines. This has allowed for the study of mGlu receptor subtypes in isolation, where the properties of a single subtype can be elucidated in the absence of other receptors. However, this can be problematic in that the loss of the native cellular environment can alter the normal regulation of the receptor. For instance, we and others have shown that the study of recombinant glutamate receptors in expression systems can be compromised due to the presence of glutamate in the extracellular medium (Thomsen *et al.*, 1994; Desai *et al.*, 1995). In the case of mGlu receptors coupled to phosphoinositide hydrolysis, the presence of glutamate in the extracellular medium can result in desensitization of the receptor (Catania *et al.*, 1991; Bessho *et al.*, 1993; Desai *et al.*, 1995). We have previously characterized human mGlu1α in a non-neuronal system in which the receptor was co-expressed with the rat GLAST transporter (Desai *et al.*, 1995). The presence of GLAST in these cells keeps glutamate levels low; therefore, we hypothesized that glutamate uptake by GLAST prevents chronic activation and subsequent desensitization of HmGlu1α. This system could therefore be manipulated both at the level of the GLAST transporter and at the level of the receptor in order to study HmGlu1α receptor function and to characterize desensitization in this receptor subtype.

In the present studies, the time-course and pharmacological properties of agonist-induced desensitization of HmGlu1α were characterized. We observed that agonist stimulation of HmGlu1α-mediated phosphoinositide hydrolysis in RGT/HmGlu1α cells was maximally inhibited upon 12 h pretreatment with direct-acting mGlu receptor agonists. This effect could be reversed by washout of agonists for 12 h after eliciting desensitization. However, pretreatment with *trans*-PDC required 24 h for maximal desensitization of HmGlu1α. This is

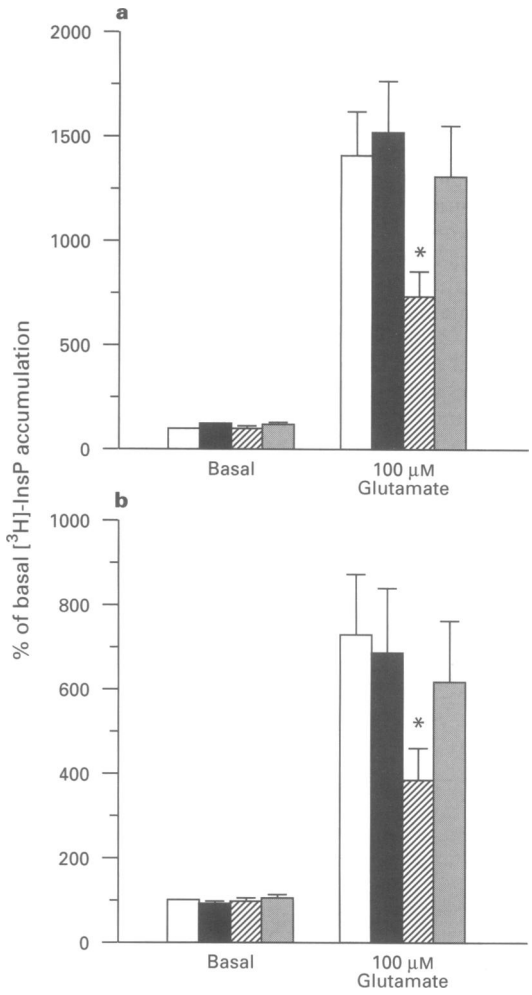

Figure 5 Effect of MCPG on desensitization of glutamate-stimulated phosphoinositide hydrolysis in RGT/HmGlu1α cells pretreated with 3,5-DHPG (a) or 1S,3R-ACPD (b). Cells were pretreated with water vehicle (control, open columns), 1 mM MCPG (solid columns), agonist (100 μM DHPG, a; 300 μM ACPD, b) (hatched columns), or MCPG plus agonist (stippled columns) for 24 h. Measurement of 100 μM glutamate-stimulated phosphoinositide hydrolysis was conducted as in Figure 1. In experiments using 3,5-DHPG as the agonist, each column represents a mean (with s.e.mean) of 6 experiments, except the basal value for 1 mM MCPG which represents a mean of 5 experiments, each done in triplicate. In experiments using 1S,3R-ACPD as the desensitizing agonist, each column represents a mean (with s.e.mean) of 3 separate experiments, each done in triplicate. $^*P<0.05$ when compared to control value for 100 μM glutamate-stimulated phosphoinositide hydrolysis.

presumably due to the fact that desensitization of HmGlu1α by *trans*-PDC involves a two-step process where the accumulation of glutamate in the extracellular medium is followed by activation of the receptor. When examining the pharmacology of activating this receptor, we found that the mGlu receptor antagonist, MCPG, greatly inhibits glutamate-stimulated phosphoinositide hydrolysis in RGT/HmGlu1α cells at the concentration used to block desensitization in the present studies (Desai *et al.*, 1995). The desensitization elicited by both *trans*-PDC and direct-acting mGlu receptor agonists was prevented by co-application of MCPG. These data support the hypothesis that desensitization of HmGlu1α subsequent to GLAST inhibition or induced by direct-acting agonists is mediated by activation of the HmGlu1α receptor.

Interestingly, desensitization of HmGlu1α did not induce greater than 50–60% inhibition of agonist-stimulated phosphoinositide hydrolysis. In contrast, agonist-induced (Catania *et al.*, 1991) or phorbol ester-induced (Ambrosini & Meldolisi,

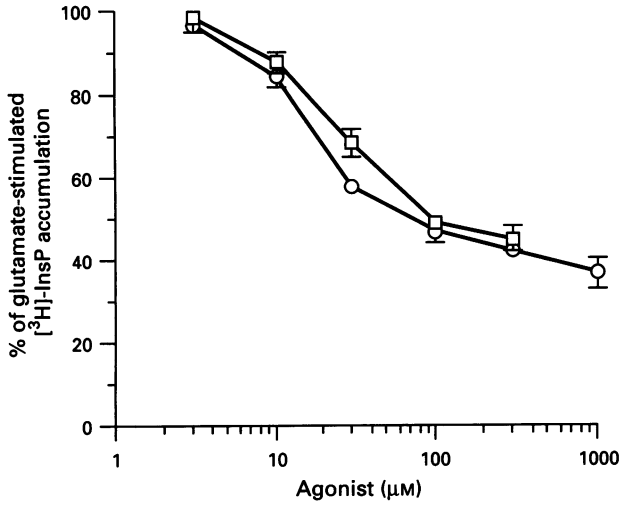

Figure 6 Concentration-effect curves for desensitization of RGT/HmGlu1α receptor coupling by 3,5-DHPG (□) or 1S,3R-ACPD (○). Cells were pretreated with various concentrations of agonists or water vehicle for 24 h. Measurement of 100 μM glutamate-stimulated phosphoinositide hydrolysis was conducted as in Figure 1. Each point represents a mean (with s.e.mean) of 3 separate experiments, each done in triplicate.

Table 1 Potency of mGlu receptor agonists in desensitizing versus activating HmGlu1α receptor coupling to phosphoinositide hydrolysis

mGlu receptor agonist	*EC_{50} (μM) receptor activation*	*EC_{50} (μM) receptor desensitization*
3,5-DHPG	10.0 ± 1.2[a] (n=6)	20.9 ± 1.9 (n=3)
1S,3R-ACPD	36.1 ± 3.4[a] (n=6)	30.0 ± 9.6 (n=3)

Values are mean ± s.e.mean. [a]Desai *et al.* (1995).

1989) desensitization of mGlu receptors in cultured neurones resulted in almost complete inhibition of agonist-stimulated phosphoinositide hydrolysis. A number of reasons may explain the lack of full inhibition of HmGlu1α-mediated phosphoinositide hydrolysis in the present studies. First, recombinant receptors in cell lines lack many of the regulatory elements that would normally exist in their cellular environment. For instance, desensitization of mGlu receptors has been shown to result in decreased expression of mGlu1α mRNA in cultured neurones (Favaron *et al.*, 1992; Bessho *et al.*, 1993). Such a mechanism could result in a loss of receptors from the cell surface. However, modulation of mRNA levels requires regulatory elements that are deleted when the coding sequence of the receptor clone is excised from its native environment and inserted into an expression vector. As a result, regulation of mRNA levels would not be expected to contribute to HmGlu1α desensitization in the RGT/HmGlu1α cell line. Previous studies have suggested that long-term activation of PKC by phorbol esters results in down-regulation of the enzyme and subsequent sensitization of receptor-mediated phosphoinositide hydrolysis (Hepler *et al.*, 1988). It is therefore possible that long-term pretreatment of RGT/HmGlu1α cells with agonist results in a counteractive effect in which PKC down-regulation decreases the degree of desensitization observed. However, this is not likely to be the case since agonist-induced desensitization of HmGlu1α was increased, rather than decreased, with time. Furthermore, we did not observe agonist-induced desensitization of phosphoinositide hydrolysis prior to 6 h incubation with mGlu receptor agonists (data not shown).

Based on the time course observed here for agonist-induced desensitization, it is possible that desensitization of HmGlu1α in the RGT cells involves a PKC-independent pathway. The time-course for desensitization of HmGlu1α described here was found to differ significantly from published reports on desensitization of rat mGlu1α. For instance, in mGlu1α-expressing BHK cells, rapid receptor phosphorylation could be elicited by application of mGlu receptor agonists or phorbol esters; and phosphorylation by either compound could be inhibited by the PKC inhibitor Ro318220 (Alaluf *et al.*, 1995). Furthermore, in cultured cerebellar granule cells, agonist-induced desensitization of mGlu receptor-mediated phosphoinositide hydrolysis was found to occur with $t1/2$ of 30 min (Catania *et al.*, 1991). The authors found that this rapid desensitization of mGlu receptor-mediated phosphoinositide hydrolysis may involve PKC since desensitization following 30 min pretreatment with agonist was inhibited by PKC inhibitors, whereas desensitization induced by 6 h agonist pretreatment was not blocked by PKC inhibitors (Catania *et al.*, 1991).

Another explanation for the long time-course for induction of desensitization in RGT/HmGlu1α cells may involve the presence of spare receptors. Previous studies of phosphoinositide hydrolysis-coupled muscarinic receptors have shown that desensitization of the receptors results in a decline in receptor binding sites before inhibition of receptor-mediated phosphoinositide hydrolysis is detected (Thompson & Fisher, 1990; Abdallah & El-Fakahany, 1991). If there is a receptor reserve, loss of receptor function would not necessarily correlate with loss of receptor coupling. However, in the present studies, a comparison of the EC_{50} values for agonist-stimulated phosphoinositide hydrolysis and agonist-induced desensitization of HmGlu1α revealed that these values are not appreciably different from each other (see Table 1). The question of spare receptors in our cell line could be better addressed with ligand binding studies to determine receptor number and occupancy; however, no selective ligands that allow measurement of HmGlu1α receptor binding are yet available.

It is not clear which mechanism or combination of mechanisms may play a role in desensitization in RGT/HmGlu1α cells. A number of putative serine/threonine phosphorylation sites are present on rat (Masu *et al.*, 1991) and human (Desai *et al.*, 1995) mGlu1α, and phosphorylation of these sites by PKC may be involved in receptor desensitization. Alternatively, since phosphorylation of the receptor is associated with rapid desensitization, other factors such as receptor internalization and down-regulation may play a role in long-term desensitization. This mechanism has been best described for β adrenoceptors (for review, see Hausdorff *et al.*, 1990).

In summary, desensitization of recombinant human mGlu1α in a non-neuronal cell line (RGT cells) involves mechanisms in which the receptor can be regulated by direct activation or by manipulation of glutamate transporter activity. In both cases evidence was provided that desensitization of HmGlu1α was ultimately mediated by activation of the human mGlu1α receptor. Since glutamate uptake sites and glutamate receptors are also co-expressed in the synapse, these observations may also be of physiological/pathological significance. However, further studies will be needed to determine which cellular mechanisms are involved in the desensitization of HmGlu1α in these cells and the relevance of these observations to the *in situ* environment where these receptors are normally expressed.

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